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(54) Title: REPERFUSION THERAPY USING ANTIBODIES TO L-SELECTIN

(57) Abstract

The invention provides methods for prevention or treatment of reperfusion injury in a patient. In these methods, a therapeutically effective dose of an agent that specifically binds to L-selectin is administered. The agent functions by inhibiting adherence of leukocytes, particularly neutrophils, to endothelial cells. In preferred methods, the agent is a human or humanized antibody specific for L-selectin.

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REPERFUSION THERAPY USING ANTIBODIES TO L-SELECTIN

This invention relates generally to therapeutic methods using anti-L-selectin antibodies for preventing ischemia/reperfusion induced tissue damage.

BACKGROUND OF THE INVENTION

Inflammation is a response of vascularized tissues to infection or injury and is effected by adhesion of leukocytes to the endothelial cells of blood vessels and their infiltration into the surrounding tissues (Harlan, *Blood* 65:513-25 (1985)) (which is hereby incorporated by reference in its entirety for all purposes). In normal inflammation, the infiltrating leukocytes phagocytize invading organisms or dead cells, and play a role in tissue repair and the immune response. However, in pathologic inflammation, infiltrating leukocytes release toxic mediators that can cause serious or fatal damage. *See, e.g.*, Hickey, *Psychoneuroimmunology II* (Academic Press 1990) (which is hereby incorporated by reference in its entirety for all purposes).

The attachment of leukocytes to endothelial cells is effected via specific interaction of cell-surface ligands and receptors, termed adhesion molecules, on endothelial cells and leukocytes. *See, generally,* Springer, *Nature* 346:425-433 (1990). Adhesion molecules include integrins, selectins, and immunoglobulin (Ig) superfamily members (*see* Springer, *supra*, Osborn, *Cell* 62:3 (1990); Hynes, *Cell* 69:11 (1992), all of which are incorporated herein by reference in their entirety for all purposes).

Integrins are heterodimeric transmembrane glycoproteins consisting of an α chain (120-180 kDa) and a β chain (90-110 kDa), generally having short cytoplasmic domains. The three known integrins, LFA-1, Mac-1 and P150,95, have different alpha subunits, designated CD11a, CD11b and CD11c, and a common beta subunit designated CD18. LFA-1 ($\alpha_L\beta_2$) is expressed on lymphocytes, granulocytes and

monocytes, and binds predominantly to an Ig-family member counter-receptor termed ICAM-1 (and perhaps to a lesser extent ICAM-2). ICAM-1 is expressed on many cells, including leukocytes and endothelial cells, and is up-regulated on vascular endothelium by cytokines such as TNF and IL-1. Mac-1 ($\alpha_M\beta_2$) is distributed on neutrophils and monocytes, and also binds to ICAM-1 (and possibly ICAM-2). The third $\beta 2$ integrin, P150,95 ($\alpha_X\beta_2$), is also found on neutrophils and monocytes.

The selectins, previously known as LECCAMs, consist of L-selectin (also called LECAM-1, Mel-14 or LAM-1), E-selectin (also called ELAM-1) and P-selectin (also called GMP140 or PADGEM). E-selectin and P-selectin are both induced on endothelial cells in response to cytokines. E-selectin is preferentially expressed in cutaneous sites of inflammation and may serve as an adhesion molecule for skin-homing T cells in inflammatory response. (Picker et al., *Nature* 349:796 (1991), which is incorporated by reference in its entirety for all purposes). E-selectin binds the carbohydrate group sialyl Lewis x (sLex) (Lowe et al., *Cell* 63:475 (1990)), which is incorporated by reference in its entirety for all purposes). L-selectin is expressed on lymphocytes and neutrophils. The cDNA sequence of human L-selectin has been published (EP 386,906). It has also been reported that L-selectin contains a sialyl Lewis group.

A number of mouse antibodies to L-selectin have been produced. *See* Kishimoto, *Proc. Natl. Acad. Sci. USA* 87:2244-2248 (1990); Kishimoto et al., *Blood* 78:805-811 (1991); Tedder et al., WO 93/02698, WO 93/00111 (each of which is incorporated by reference in its entirety for all purposes). Antibody binding studies have indicated that L-selectin specifically interacts with, *inter alia*, E-selectin and P-selectin (Picker et al., *Cell* 66:921 (1991), which is incorporated by reference in its entirety for all purposes). These analyses have also suggested possible roles for L-selectin as (1) a T-cell homing receptor for the high endothelial venules of peripheral lymph nodes, and (2) a neutrophil adhesion molecule having affinity for endothelial cells (Hallmann et al., *Biochem. Biophys. Res. Commun.* 174:236

(1991)), which is incorporated by reference in its ntirety for all purposes).

The identification of integrins and selectins as adhesive molecules effecting binding of neutrophils to endothelial cells suggests that these molecules might be potential therapeutic targets in treatment of inflammatory diseases. However, it is likely that the nature and degree of expression of these molecules varies for different cell subtypes and inflammatory stimuli. Thus, particular agents targeted against these various molecules are likely to be effective only for certain types of inflammatory conditions.

Ischemia/reperfusion injury is an inflammatory condition that occurs on restoring blood flow to organs suffering from an obstructed supply causing ischemia (oxygen deprivation). Unless rapidly relieved by reperfusion, ischemia causes death of surrounding cells, and eventually, death of a whole organ or patient. However, accumulating evidence suggests that reperfusion may itself exert deleterious effects on surrounding tissue. The deleterious effects of reperfusion are believed to result at least in part from an inflammatory response mediated by activated neutrophils in the restored blood flow.

Monoclonal antibodies that block the interaction of integrins with ICAM-1 have been reported to be somewhat successful in reducing myocardial tissue damage in animal models of the ischemia-reperfusion condition. These antibodies were specific either for CD18 (the common subunit of integrins) or against ICAM-1. Ma et al, *J. Clin. Invest.* 88:1237-1243 (1991); Ma et al, *Circulation* 86:937-46 (1992) (each of which is incorporated by reference in its entirety for all purposes). However, it is possible that other adhesion molecules besides the integrins and their receptors play a role in ischemia-reperfusion injury. Agents targeted against such adhesion molecules would considerably augment the repertoire of possible therapeutic treatments. Combinati ns of therapeutic agents may be more effective than either agent alone. Moreover, the availability of alternative agents

provides treatment options should any one agent induce intolerance in a particular patient.

Based on the foregoing, it is apparent that a need exists for additional and more effective therapeutic agents and methods for treatment of ischemia-reperfusion injury. The present invention fulfills this and other needs.

SUMMARY OF THE INVENTION

The invention provides methods for prevention or treatment of reperfusion injury in a patient. In these methods, a therapeutically effective dose of an agent that specifically binds to L-selectin is administered. The agent may be administered directly to the patient or to an isolated organ awaiting transplant into the patient. The agent functions by inhibiting adherence of leukocytes, particularly neutrophils, to endothelial cells. The agents include antibodies that specifically bind to L-selectin. Usually, the methods are performed using monoclonal antibodies. Humanized or human antibodies are preferred. Some antibodies compete with an antibody designated hu DREG-200 for specific binding to L-selectin. The methods are particularly useful for treating patients suffering from myocardial ischemia. In some methods, administration of an agent specific for L-selectin is combined with administration of a therapeutically effective dose of a second agent. For example, in some methods, the second agent is a thrombolytic agent and in other methods, the agent is an antibody specific for CD18.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Amino acid sequences of the mature light chain (A) and heavy chain (B) variable regions of the mouse DREG-200 antibody (upper lines) and humanized DREG-200 antibody (hu DREG-200) (lower lines). The three CDRs in each chain are underlined. Residues in the framework that have been replaced with mouse amino acids or typical human amino acids in the humanized antibody are double underlined.

Figure 2: Inhibition of *in vitro* binding of neutrophils to ischemia-reperfusion-activated endothelial cells by monoclonal antibody hu DREG-200. LCX, left circumflex coronary artery; LAD, left anterior descending coronary artery.

Figure 3: *In vivo* protection of myocardial tissue from ischemia-reperfusion injury by monoclonal antibody hu DREG-200. Tissue damage was assayed by staining with nitroblue tetrazolium.

Figure 4: *In vivo* protection of myocardial tissue from ischemia-reperfusion injury by monoclonal antibody hu DREG-200. Tissue damages was assayed by determining plasma creatine kinase activity (CK). O = occlusion, R = reperfusion.

Figure 5: Inhibition of neutrophil accumulation at ischemic endothelial cells, by hu DREG-200, during reperfusion. Neutrophils were quantified from MPO activity.

Figure 6: Improved mechanical performance of the heart during reperfusion of ischemic myocardial tissue conferred by hu DREG-200 treatment. Data are expressed as percentages of initial values.

Figure 7: Reduction of coronary endothelial cell dysfunction in ischemic-reperfused tissue by treatment with hu DREG-200.

DEFINITIONS

The term "substantial identity" or "substantial homology" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity or more (*e.g.*, 99 percent sequence identity). Preferably, residue positions which are not identical differ by conservative amino acid substitutions.

For purposes of classifying amino acids substitutions as conservative or nonconservative, amino acids are grouped as follows: Group I (hydrophobic sidechains): norleucine, met, ala, val, leu, ile; Group II (neutral

hydrophilic side chains): cys, ser, thr; Group III (acidic side chains): asp, glu; Group IV (basic side chains): asn, gln, his, lys, arg; Group V (residues influencing chain orientation): gly, pro; and Group VI (aromatic side chains): trp, tyr, phe. Conservative substitutions involve substitutions between amino acids in the same class. Non-conservative substitutions constitute exchanging a member of one of these classes for another.

Amino acids from the variable regions of the mature heavy and light chains of immunoglobulins are designated Hx and Lx respectively, where x is a number designating the position of an amino acid according to the scheme of Kabat, *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD (1987) and (1991) (incorporated by reference in their entirety for all purposes). Kabat lists many amino acid sequences for antibodies for each subclass, and lists the most commonly occurring amino acid for each residue position in that subclass. Kabat uses a method for assigning a residue number to each amino acid in a listed sequence, and this method for assigning residue numbers has become standard in the field. Kabat's scheme is extendible to other antibodies not included in his compendium by aligning the antibody in question with one of the consensus sequences in Kabat. The use of the Kabat numbering system readily identifies amino acids at equivalent positions in different antibodies. For example, an amino acid at the L50 position of a human antibody occupies the equivalent position to an amino acid position L50 of a mouse antibody.

From N-terminal to C-terminal, both light and heavy chains comprise the domains FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4. The assignment of amino acids to each domain is in accordance with the structural definition of Kabat (1987 and 1991), *supra*, or Chothia & Lesk, *J. Mol. Biol.* 196:901-917 (1987); Chothia et al. *Nature* 342:878-883 (1989) (incorporated by reference in their entirety for all purposes).

DETAILED DESCRIPTION

The invention provides methods of reperfusion therapy and pharmaceutical compositions for use in the same. The pharmaceutical compositions contain agents that specifically bind to L-selectin. The agents function by preventing leukocytes, particularly neutrophils, bearing L-selectin on their surface from interacting with endothelial cells, and thereby initiating an inflammatory response.

I. Active Agents

The invention provides antibodies specific for L-selectin, and other molecules, such as miniproteins, that have a similar binding specificity, for use in therapeutic methods and pharmaceutical compositions.

A. Antibodies

1. General Characteristics

The basic antibody (or immunoglobulin) structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kDa) and one "heavy" chain (about 50-70 kDa). The NH_2-terminus of each chain begins a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The COOH part of each chain defines a constant region primarily responsible for effector function.

Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, and define the antibody's isotype as IgG, IgM, IgA, IgD and IgE, respectively. Within light and heavy chains, the variable and constant regions are joined by a "J" region of about 12 or more amino acids, with the heavy chain also including a "D" region of about 10 more amino acids. (*See, generally, Fundamental Immunology*, Paul, W., Ed., Chapter 7, pp. 131-166, Raven Pr ss, N.Y. (1984), which is incorporated by reference in its entirety for all purp ses.)

Th variable regions of ach light/heavy chain pair form the antibody binding site. The chains all exhibit the

same general structure of relatively conserved framework regions joined by three hypervariable regions, also called Complementarity Determining Regions or CDRs (*see "Sequences of Proteins of Immunological Interest,"* Kabat, E., et al., U.S. Department of Health and Human Services (1987); and Chothia and Lesk, *J. Mol. Biol.*, 196:901-917 (1987), which are incorporated by reference in their entirety for all purposes). The CDRs from the two chains of each pair are aligned by the framework regions, enabling binding to a specific epitope.

The term "antibody" or "immunoglobulin" is used generically to refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Thus, the term "antibody" includes not only intact antibodies, but, for each intact antibody discussed, a variety of fragments having the same or similar binding specificity as the intact antibody. Fragments include Fv, Fab, and $(Fab')_2$ as well as bifunctional hybrid antibodies (*e.g.*, Lanzavecchia et al., *Eur. J. Immunol.* 17:105 (1987)) and single chains (*e.g.*, Huston et al., *Proc. Natl. Acad. Sci. U.S.A.*, 85:5879-5883 (1988) and Bird et al., *Science*, 242:423-426 (1988), which are incorporated by reference in their entirety for all purposes). (*See, generally*, Hood et al., *Immunology*, Benjamin, N.Y., 2nd ed. (1984), Harlow and Lane, *Antibodies. A Laboratory Manual*, Cold Spring Harbor Laboratory (1988) and Hunkapiller and Hood, Nature, 323:15-16 (1986), which are incorporated by reference in their entirety for all purposes).

2. Binding Characteristics

The agent of the invention should bind to an epitope of L-selectin so as to inhibit adhesion of neutrophils to endothelial cells. Many antibodies are targeted against L-selectin epitope(s) that are responsible for (i.e., participat directly in) binding of leukocytes to endothelial cells. However, other agents are targeted against neighboring epitopes and function by steric hindrance of the epitope(s)

that directly participate in binding. The term "L-selectin," encompasses a human L-selectin polypeptide having the amino acid sequence presented in Tedder, EPA 386,906 (1990), allelic and nonallelic variants thereof, cognate variants, such as the mouse MEL-14 receptor, and natural and induced mutants of any of these. L-selectin is a type I membrane receptor having a molecular weight of about 80-85 kDa. The intact polypeptide has been subdivided into the following domains: a lectin domain, an EGF-like domain, a first short consensus domain, a second consensus repeat domain, a leader sequence domain, a transmembrane domain and a phosphorylation domain. *See* Tedder et al, WO 93/02698.

One example of an antibody having the required binding specificity is the antibody DREG-200. *See* Kishimoto et al., *Proc. Natl. Acad. Sci., supra* (mouse antibody) and Co et al., copending application, USSN 07/983,946 (humanized antibody, hereinafter sometimes "hu DREG-200") (which are hereby incorporated by reference in their entirety for all purposes). Other antibodies having the same or similar epitope specificity can be isolated by competitive binding experiments with DREG-200 (mouse or humanized). In competitive binding experiments, the L-selectin molecule can be expressed on the surface of neutrophils or from transformed COS cells. Alternatively, purified recombinant L-selectin, or a binding domain thereof, can be immobilized to a solid support.

Antibodies having the required epitope specificity can also be identified by direct screening for the capacity to block neutrophil-endothelial cell interaction. A simple visual assay for detecting such interaction has been described by Kishimoto (1991), *supra*. Briefly, monolayers of human umbilical vein cells are stimulated with IL-1. Neutrophils, with or without pretreatment with the antibody under test, are added to the monolayer under defined conditions, and the number of adhering neutrophils is d termined microscopically. In one method, the neutrophils are obtained fr m human leukocyte adhesion deficient pati nts. *See* Anderson et al., *Ann. Rev. Med.* 38:175 (1987). The neutrophils from such

patients lack integrin receptors, whose binding to neutrophils might obscur the effects of blocking L-selectin binding.

An alternative and preferred method of screening for suitable antibodies is described in Example 2. In this method, endothelial cells for use in the adhesion assay are activated *in vivo* by ischemia-reperfusion of laboratory animals. Tissue bearing endothelial cells is extracted and incubated with labelled neutrophils, with and without pretreatment with test antibody, *in vitro*. Neutrophils attaching to endothelial cells are detected by fluorescence microscopy.

An alternative approach for identifying antibodies having the desired specificity is to map the L-selectin amino acid residues required for binding to an exemplary antibody, such as DREG-200 (mouse or humanized), known to have appropriate specificity. The epitope bound by DREG-200 or any other anti-L-selectin antibody is determined by providing a family of fragments containing different amino acid segments from L-selectin. Each fragment typically comprises at least 4, 6, 8, 10, 20, 50 or 100 contiguous amino acids. Collectively, the family of polypeptide covers much or all of the amino acid sequence of a full-length L-selectin peptide. Members of the family are tested individually for binding to, *e.g.*, the DREG-200 antibody (mouse or humanized) by, *e.g.*, immunoprecipitation or Western blotting. The smallest fragment that can specifically bind to the antibody under test delineates the amino acid sequence of the epitope recognized by the antibody. Alternatively, epitopes can be identified by protein footprinting of L-selectin polypeptides. In this technique, an L-selectin polypeptide is allowed to bind to an antibody and then exposed to a protease. The residues of L-selectin binding to the monoclonal antibody are protected from proteolytic degradation, and identified by amino acid sequencing. L-selectin fragments consisting essentially of the epitope(s) identified by the above analyses, are suitable immunogens to be used in the methods for producing antibodies discussed *infra*.

The strength of antibody binding to its antigen provides an indication of therapeutic potential, with str nger binding likely to be more useful. Usually, the antibodies will have an affinity of at least about 10^6 M^{-1}, more usually at least about 10^8 M^{-1} and preferably at least about 10^9 M^{-1} for L-selectin. For example, mouse DREG-200 binds with an affinity of about 10^8 M^{-1}. Other preferred antibodies will have binding affinities within a factor of about three of that of mouse or hu DREG-200, or higher.

3. Production of Antibodies

a. Nonhuman Antibodies

The production of non-human monoclonal antibodies, e.*g.*, murine, rat and so forth, is well known and may be accomplished by, for example, immunizing the animal with a preparation containing an L-selectin polypeptide or an immunogenic fragment thereof. Particularly, useful as immunogens are cells naturally expressing or stably transfected with recombinant L-selectin and expressing L-selectin receptors on their cell surface. Antibody-producing cells obtained from the immunized animals are immortalized and screened for the production of an antibody which binds to L-selectin. *See* Harlow & Lane, *Antibodies, A Laboratory Manual* (C.S.H.P. NY, 1988) (incorporated by reference in its entirety for all purposes).

A number of murine antibodies to L-selectin have already been produced. These include mouse DREG-55, -56, -110, -152 and 200 discussed by Kishimoto et al, *Proc. Natl. Acad Sci. USA* 87:2244-2248 (1990) and anti-LAM-1, -2, -4, -5, -6, -8, -10, 11, -14 and -15 discussed by Tedder et al, WO 93/02698. The DREG-55 and -56 antibodies are likely to be effective in the present methods because they have been shown to block binding of neutrophils to endothelial cells *in vitro*.

Nonhuman antibodies produced by the above methods are generally not preferred for use in therapeutic methods. H wever, such antibodies are useful for generating humanized and, in some instances, human antibodies by th proc dures discussed *infra*.

b. Humanized and Human Antibodies

These antibodies have at least three potential advantages over nonhuman antibodies, such as mouse antibodies, for use in human therapy:

1) because the effector portion is human, it may interact better with the other parts of the human immune system (*e.g.*, destroy the target cells more efficiently by complement-dependent cytotoxicity (CDC) or antibody-dependent cellular cytotoxicity (ADCC));
2) the immune response against human or humanized antibodies should be minimal or absent;
3) human and humanized antibodies are expected to have a longer half life than mouse antibodies in the human circulation (*see* Shaw et al, *J. Immunol.* 138:4534-4538 (1987)).

(1) Humanized Antibodies

Humanized immunoglobulins are produced by linking the CDR regions of non-human antibodies known to have useful properties to human constant regions by recombinant DNA techniques. *See* Queen et al., *Proc. Natl. Acad. Sci. USA* 86:10029-10033 (1989) and WO 90/07861 (incorporated by reference in their entirety for all purposes). The humanized immunoglobulins have variable region framework residues substantially from a human immunoglobulin (termed an acceptor immunoglobulin) and complementarity determining regions substantially from a nonhuman immunoglobulin, *e.g.*, the mouse DREG-200 antibody (referred to as the donor immunoglobulin). The constant region(s), if present, are also substantially from a human immunoglobulin.

The human variable domains are usually chosen from human antibodies whose framework sequences exhibit a high degree of sequence identity with the murine variable regi n domains from which the CDRs were derived. The heavy and light chain variable framework r gions can be derived from the same or different human antibody s quences. Indeed, the heavy and

light chain framework regions can each be derived from more than ne human antibody. The human antibody sequences can be the sequences of naturally occurring human antibodies r can be consensus sequences of several human antibodies. *See* Carter et al., WO 92/22653 (1992) (incorporated by reference in its entirety for all purposes).

The unnatural juxtaposition of murine CDR regions with human variable framework region can result in unnatural conformational restraints, which, unless corrected by substitution of certain amino acid residues, lead to loss of binding affinity. The selection of amino acid residues for substitution is determined, in part, by computer modelling. Computer hardware and software for producing three-dimensional images of immunoglobulin molecules are widely available. In general, molecular models are produced starting from solved structures for immunoglobulin chains or domains thereof. The chains to be modelled are compared for amino acid sequence similarity with chains or domains of solved three dimensional structures, and the chains or domains showing the greatest sequence similarity is/are selected as starting points for construction of the molecular model. The solved starting structures are modified to allow for difference between the actual amino acids in the immunoglobulin chains or domains being modelled, and those in the starting structure. The modified structures are then assembled into a composite immunoglobulin. Finally, the model is refined by energy minimization and by verifying that all atoms are within appropriate distances from one another and that bond lengths and angles are within chemically acceptable limits. Additional models can be constructed representing the structure when further amino acid substitutions to be discussed *infra*, are introduced.

In general, substitution of human amino acid residues with murine should be minimized, because introduction of murine residues increases the risk of the antibody eliciting a HAMA response in humans. Amino acids ar selected for substitution based on their possible influence on CDR conformation and/or binding to antigen. Investigation of such

possible influences is by modelling, examination of the characteristics of the amino acids at particular locations, or empirical observation of the effects of substitution or mutagenesis of particular amino acids.

When an amino acid differs between a mouse variable framework region and an equivalent human variable framework region, the human framework amino acid should usually be substituted by the equivalent mouse amino acid if it is reasonably expected that the amino acid:

(1) noncovalently binds antigen directly;

(2) is adjacent to a CDR region or otherwise interacts with a CDR region (*e.g.*, is within about 4-6 Å of a CDR region).

Other candidates for substitution are acceptor human framework amino acids that are unusual for a human immunoglobulin at that position. These amino acids can be substituted with amino acids from the equivalent position of more typical human immunoglobulins. Alternatively, amino acids from equivalent positions in the mouse donor antibody can be introduced into the human framework regions when such amino acids are typical of human immunoglobulins at the equivalent positions.

In general, substitution of all or most of the amino acids fulfilling the above criteria is desirable. Occasionally, however, there is some ambiguity about whether a particular amino acid meets the above criteria, and alternative variant immunoglobulins are produced, one of which has that particular substitution, the other of which does not.

Usually the CDR regions in humanized antibodies are substantially identical, and more usually, identical to the corresponding CDR regions in the mouse donor antibody (e.g., mouse DREG-200). Occasionally, however, it is desirable to change one of the residues in a CDR region, for example, to create a resemblance to the binding site of a ligand of L-selectin. Although not usually desirable, it is sometimes possible to make one or more conservative amino acid substitutions of CDR residues without appreciably affecting the binding affinity of the resulting humanized immunoglobulin.

Other than for the specific amino acid substitutions discussed above, the framework regions of humanized immunoglobulins are usually substantially identical, and more usually, identical to the framework regions of the human antibodies from which they were derived. However, in some embodiments the framework regions can vary from the native sequences at the primary structure level by several amino acid substitutions, terminal and intermediate additions and deletions, and the like. Stereoisomers (*e.g.*, D-amino acids) of the twenty conventional amino acids, unnatural amino acids such as α,α-disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids may also be suitable components for polypeptides of the present invention. Of course, many of the amino acids in the framework region make little or no direct contribution to the specificity or affinity of an antibody. Thus, many individual conservative substitutions of framework residues can be tolerated without appreciable change of the specificity or affinity of the resulting humanized immunoglobulin. However, in general, such substitutions are undesirable. Modifications of the genes may be readily accomplished by a variety of well-known techniques, such as site-directed mutagenesis (*see* Gillman & Smith, *Gene* 8:81-97 (1979) and Roberts et al., *Nature* 328:731-734 (1987), both of which are incorporated by reference in their entirety for all purposes).

The amino acids sequences of the variable regions of an exemplary humanized antibody, designated hu DREG-200, are shown in Fig. 1. This antibody was constructed by fusing the CDR regions of the murine DREG-200 antibody with the framework regions of the human Eu antibody. Human light chain residues at positions L87, L54, L66, L76 and L93, were replaced with corresponding murine residues. Human heavy chain residues at positions H93, H95, H98, H111, H112, H115, H30, H98, H111, H27, H48, and H72 were replaced with corresponding murine residues. Human h avy chain residue H116 was replac d with an amino acid more typical of human immunoglobulin heavy chains at that position.

The variable segments of humanized antibodies produced as described *supra*, are typically link d to at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. Human constant region DNA sequences can be isolated in accordance with well-known procedures from a variety of human cells, but preferably immortalized B-cells (*see* Kabat, *supra*, and WO87/02671). Ordinarily, the antibody will contain both light chain and heavy chain constant regions. The heavy chain constant region usually includes CH1, hinge, CH2, and CH3 regions.

The humanized antibodies include antibodies having all types of constant regions, including IgM, IgG, IgD, IgA and IgE, and any isotype, including IgG1, IgG2, IgG3 and IgG4. When cytotoxic activity is not desirable, the constant domain may be of the IgG4 class.

(2) Human Antibodies

Human antibodies can now be produced by a variety of techniques. (*See, e.g.*, Larrick et al., U.S. Patent No. 5,001,065, for review, which is hereby incorporated by reference in its entirety for all purposes). Preferred techniques for generation of human anti-L-selectin antibodies are discussed below.

(a) *Trioma Methodology*. The basic approach and an exemplary cell fusion partner, SPAZ-4, for use in this approach have been described by Oestberg et al., *Hybridoma* 2:361-367 (1983); Oestberg, U.S. Patent No. 4,634,664; and Engleman et al., US Patent 4,634,666 (each of which is incorporated by reference in its entirety for all purposes). The antibody-producing cell lines obtained by this method are called triomas, because they are descended from three cells--two human and one mouse. Initially, a mouse myeloma line is fused with a human B-lymphocyte to obtain a non-antibody-producing xenogeneic hybrid cell, such as th SPAZ-4 cell line described by Oestberg, *supra*. Th xenogeneic cell is th n fused with an immunized human B-lymphocyt to obtain an antibody-producing trioma cell line. Triomas have been found

to produce antibody more stably than ordinary hybridomas made fr m human cells.

The immunized B-lymphocytes are obtained from the blood, spleen, lymph nodes or bone marrow of a human donor. *In vivo* immunization of a living human with L-selectin is usually undesirable because of the risk of initiating a harmful response. Thus, B-lymphocytes are usually immunized *in vitro* with an L-selectin polypeptide, an antigenic fragment thereof or a cell bearing either of these. If antibodies against a specific antigen or epitope are desired, it is preferable to use that antigen or epitope thereof for *in vitro* immunization. B-lymphocytes are typically exposed to antigen for a period of 7-14 days in a media such as RPMI-1640 (*see* Engleman, *supra*) supplemented with 10% human plasma.

The immunized B-lymphocytes are fused to a xenogeneic hybrid cell such as SPAZ-4 by well known methods. For example, the cells are treated with 40-50% polyethylene glycol of MW 1000-4000, at about 37 degrees, for about 5-10 min. Cells are separated from the fusion mixture and propagated in media selective for the desired hybrids (*e.g.*, HAT or AH). Clones secreting antibodies having the required binding specificity are identified by assaying the trioma culture medium for the ability to bind to L-selectin or a fragment thereof. Triomas producing human antibodies having the desired specificity are subcloned by the limiting dilution technique and grown *in vitro* in culture medium. The trioma cell lines obtained are then tested for the ability to bind L-selectin or a polypeptide thereof.

Although triomas are genetically stable they do not produce antibodies at very high levels. Expression levels can be increased by cloning antibody genes from the trioma into one or more expression vectors, and transforming the vector into a suitable cell line for expression of recombinant immunoglobulins. *See, e.g.*, Co et al., *J. Immunol.*, 148:1149 (1992) (incorporated by reference in its entirety for all purposes).

(b) *Transgenic Non-Human Mammals*. Human antibodies against L-selectin can also be produced from non-human

transgenic mammals having transgenes encoding at least a segment of the human immunoglobulin locus. Usually, the endogenous immunoglobulin locus of such transgenic mammals is functionally inactivated. Preferably, the segment of the human immunoglobulin locus includes unrearranged sequences of heavy and light chain components. Inactivation of endogenous immunoglobulin genes and introduction of exogenous immunoglobulin genes can be achieved by targeted homologous recombination, or by the introduction of YAC chromosomes. The transgenic mammals resulting from this process are capable of functionally rearranging the immunoglobulin component sequences, and expressing a repertoire of antibodies of various isotypes encoded by human immunoglobulin genes, without expressing endogenous immunoglobulin genes. The production and properties of mammals having these properties are described in detail by, *e.g.*, Lonberg et al., WO93/12227 (1993); Kucherlapati, WO 91/10741 (1991) (each of which is incorporated by reference in its entirety for all purposes). Transgenic mice are particularly suitable. Anti-L-selectin antibodies are obtained by immunizing a transgenic nonhuman mammal, such as described by Lonberg or Kucherlapati, *supra*, with L-selectin or a fragment thereof. Monoclonal antibodies are prepared by, *e.g.*, fusing B-cells from such mammals to suitable myeloma cell lines using conventional Kohler-Milstein technology.

c. Phage Display Methods: A further approach for obtaining human anti-L-selectin antibodies is to screen a DNA library from human B cells according to the general protocol outlined by Huse et al., *Science* 246:1275-1281 (1989). Antibodies binding to L-selectin or a fragment thereof are selected. Sequences encoding such antibodies (or a binding fragments) are then cloned and amplified. The protocol described by Huse is rendered more efficient in combination with phage-display technology. *See, e.g.*, Dower et al., WO 91/17271 and McCafferty et al., WO 92/01047 (each of which is incorporated by reference in its entirety for all purposes). In these methods, libraries of phage are produced in which members display different antibodies on th ir outersurfaces.

Antibodies are usually displayed as Fv or Fab fragments. Phage displaying antibodies with a desired specificity are selected by affinity enrichment to an L-selectin polypeptide or fragment thereof.

In a variation of the phage-display method, human antibodies having the binding specificity of a selected murine antibody can be produced. *See* Winter, WO 92/20791. In this method, either the heavy or light chain variable region of the selected murine antibody (*e.g.*, mouse DREG-200) is used as a starting material. If, for example, a light chain variable region is selected as the starting material, a phage library is constructed in which members displays the same light chain variable region (i.e., the murine starting material) and a different heavy chain variable region. The heavy chain variable regions are obtained from a library of rearranged human heavy chain variable regions. A phage showing strong specific binding for L-selectin (*e.g.*, at least about 10^8 and preferably at least about 10^9 M^{-1}) is selected. The human heavy chain variable region from this phage then serves as a starting material for constructing a further phage library. In this library, each phage displays the same heavy chain variable region (i.e., the region identified from the first display library) and a different light chain variable region. The light chain variable regions are obtained from a library of rearranged human variable light chain regions. Again, phage showing strong specific binding for L-selectin are selected. These phage display the variable regions of completely human anti-L-selectin antibodies. These antibodies usually have the same or similar epitope specificity as the murine starting material (*e.g.*, mouse DREG-200).

B. Other Therapeutic Agents

Having produced an antibody having desirable properties, such as DREG-200 (mouse or humanized), other n nantib dy agents having similar binding specificity/and or affinity can be produced by a vari ty of methods. For example, Fodor et al., US 5,143,854, discuss a technique termed VLSIPS™, in which a diverse collection of short

peptides are formed at selected positions on a solid substrate. Such peptides could then be screened for binding to the L-selectin epitope r cognized by DREG-200, optionally in competition with the DREG-200 antibody (mouse or humanized). Libraries of short peptides can also be produced and screened using phage-display technology. *See, e.g.*, Devlin WO91/18980. Fragments of a ligand of L-selectin that compete with the ligand for binding to neutrophils bearing L-selectin are also suitable agents for use in the present method. Optionally, these fragments, or indeed polypeptides produced by other methods, can be variegated to achieve improved binding affinity for L-selectin. *See, e.g.*, Ladner, US 5,223,409 (which is hereby incorporated by reference in its entirety for all purposes).

II. Pharmaceutical Compositions

The pharmaceutical compositions for use in the therapeutic methods discussed *infra*, typically comprises an active agent, such as an anti-L-selectin antibody, dissolved in an acceptable carrier, preferably an aqueous carrier. Some compositions contain a cocktail of multiple active agents, for example, an anti-L-selectin antibody and a thrombolytic agent. A variety of aqueous carriers can be used, *e.g.*, water, buffered water, phosphate buffered saline (PBS), 0.4% saline, 0.3% glycine, human albumin solution and the like. These solutions are sterile and generally free of particulate matter. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example, sodium acetate, sodium chloride, potassium chloride, calcium chloride and sodium lactate. The concentration of antibody in these formulations can vary widely, *i.e.*, from less than about 0.005%, usually at least about 1% to as much as 15 or 20% by weight and will be selected primarily based on fluid volumes, viscositi s, and so forth, in accordance with the particular mode of administration selected.

Thus, a typical pharmaceutical composition for injection could be made up to contain 1 ml sterile buffered water, and 1-10 mg of immunoglobulin. A typical composition for intravenous infusion could be made up to contain 250 ml of sterile Ringer's solution, and 150 mg of antibody. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in, for example, *Remington's Pharmaceutical Science* (15th ed., Mack Publishing Company, Easton, PA, 1980), which is incorporated by reference in its entirety for all purposes. Compositions suitable for lavage or other routes will be selected according to the particular use intended.

Therapeutic agents of the invention can be frozen or lyophilized for storage and reconstituted in a suitable carrier prior to use. Lyophilization and reconstitution can lead to varying degrees of antibody activity loss (*e.g.*, with conventional immune globulins, IgM antibodies tend to have greater activity loss than IgG antibodies). Dosages may have to be adjusted to compensate.

III. Therapeutic Methods

The invention provides methods for reperfusion therapy using the therapeutic agents and pharmaceutical conditions discussed *supra*. The methods are particularly useful for therapeutic and prophylactic treatment of ischemia-reperfusion injury caused by, for example, myocardial infarction, cardiac surgery, such as coronary artery bypass or elective angioplasty, cerebral ischemic event (*e.g.*, a stroke or brain surgery), renal, splenic or hepatic ischemic events, shock (*e.g.*, hemorrhagic-shock-induced gastric mucosal injury), and the like.

The methods are particularly useful for humans, but may also be practiced on veterinary subjects. The therapeutic methods are usually applied to organs present in living subjects. However, the methods are equally applicable to dissected organs, particularly when such organs are awaiting transplant to a recipi nt pati nt.

The therapeutic agents and compositions targeted against L-selectin can also be us d in combination with agents targ ted against other molecules, particularly humanized or human antibodies reactive with different adhesion molecules. Suitable immunoglobulins include those specific for CD11a, CD11b, CD18, E-selectin, P-selectin and ICAM-1. The immunoglobulins should bind to epitopes of these adhesion molecules so as to inhibit binding of leukocytes, particularly neutrophils, to endothelial cells. The combination of therapeutic agents against L-selectin with agents against CD18 is particularly preferred. It has been reported that L-selectin and CD18 mediate sequential stages in the neutrophil-endothelial cell attachment process. *See* von Andrian et al., *Proc. Natl. Acad. Sci. USA* 88:7538 (1991); Luscinskas, *J. Immunol.* 142:2257 (1989) (each of which is incorporated herein by reference in its entirety for all purposes). Anti-CD18 antibodies are described in, *e.g.*, EPA 440,351 and US 4,797,277 (incorporated by reference in their entirety for all purposes). Thus, a combination of agents directed against both targets is likely to provide a particularly effective reperfusion therapy. Other suitable antibodies for use in combination therapies are those specific for lymphokines, such as IL-1, IL-2 and IFN-γ, and their receptors. Such antibodies serve to block activation of endothelial cells, and thereby prevent their interaction with neutrophils in an inflammatory response.

In some methods of reperfusion therapy, therapeutic agents directed against L-selectin are used in combination with thrombolytic agents. The thrombolytic agent serves to remove a blockage to the blood supply, while the anti-L-selectin agent protects against tissue injury resulting from restoration of the blood supply. Thrombolytic agents are drugs having the capacity, directly or indirectly, to stimulate dissolution of thrombi *in vivo*. Thrombolytic agents include tissue plasminog n activator (*see* EP-B 0 093 619), activase, alteplase, duteplase, silteplase, str ptokinase, anistreplase, urokinase, heparin, warfarin and coumarin. Additional thrombolytic ag nts include saruplase and vampire

bat plasminogen activator. *See* Harris, *Protein Engineering* 6 (1987) 449-458; PCT/EP 90/00194; US Patent 4,970,159) (incorporated by reference in their entirety for all purposes).

The combined use of anti-L-selectin agents and thrombolytic agents is particularly useful for treating patients with acute myocardial infarction. In conventional methods, the obstructed coronary artery may be reopened by administration of thrombolytic agents alone. These agents lyse the clot causing the obstruction, thereby restoring coronary blood flow. Alternatively, conventional methods restore blood flow by acute percutaneous transluminal coronary angioplasty (PTCA), a procedure in which an obstructed and narrowed segment of the coronary artery is dilated with a balloon. However, restoration of coronary blood flow by either of these approaches can lead to ischemia-reperfusion injury.

In the present methods, ischemia-reperfusion injury is reduced or prevented by combination of a thrombolytic agent or of PTCA with humanized or human anti-L-selectin antibodies. Antibodies are usually administered prophylactically before or at the same time as administration of thrombolytic agents or initiation of PTCA. Further doses of antibody are then often administered during and after thrombolytic or angioplastic treatment. The interval between prophylactic administration of the antibodies and initiation of thrombolytic or angioplastic treatment is usually 5-30 min, preferably 5-20 min, and most preferably 5-10 min.

Thrombolytic agents are administered to a patient in an amount sufficient to partially disperse, or prevent the formation of, thrombi and their complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose" or "efficacious dose." Amounts effective for this use will depend upon the severity of the condition, the general state of the patient, the route of administration and combination with other drugs. Often, therapeutically effective doses of thrombolytic agents and administration regimens for such agents are those approved by the FDA, for

independent uses of thrombolytic agents, *e.g.*, 100 mg of alteplase or 1.5 million IU of streptokinase.

The antibodies of the invention can also be used in conjunction with chemotherapeutic agents. Typically, the agents may include non-steroidal anti-inflammatory drugs (e.g., aspirin) and corticosteroids, but numerous additional agents (*e.g.*, cyclosporin) well-known to those skilled in the art of medicine may also be utilized.

The invention also provides methods of reperfusion therapy in which anti-L-selectin agents are conjugated to toxin moieties. In these methods, neutrophils expressing L-selectin are not merely inhibited, but eliminated, by the toxin moiety. The therapeutic agent component provides a means for delivering the toxic agent to cells expressing an L-selectin epitope. The two components can be bonded by heterobifunctional cross-linkers, *e.g.*, SPDP, carbodiimide, glutaraldehyde, or the like. The components may also be linked genetically (*see* Chaudhary et al., *Nature* 339:394 (1989), incorporated by reference in its entirety for all purposes). Other methods of producing toxin conjugates are discussed by Thorpe et al. in *Monoclonal Antibodies in Clinical Medicine* (Academic Press, 1982), pp. 168-190, which is incorporated by reference in its entirety for all purposes).

A variety of cytotoxic agents are suitable for use in immunotoxins. Cytotoxic agents can include radionuclides, such as Iodine-131 or other isotopes of iodine, Yttrium-90, Rhenium-188, and Bismuth-212 or other alpha emitters; a number of chemotherapeutic drugs, such as vindesine, methotrexate, adriamycin, and cisplatin; and cytotoxic proteins such as ribosomal inhibiting proteins like pokeweed antiviral protein, Pseudomonas exotoxin A, ricin, diphtheria toxin, ricin A chain, etc., or an agent active at the cell surface, such as the phospholipase enzymes (*e.g.*, phospholipase C). (*See, generally*, commonly assigned U.S.S.N. 07/290,968, "Chimeric Toxins," Olsnes & Phil, *Pharmac. There.*, 25:355-381 (1982), and *Monoclonal Antibodies for Cancer Det ction and Therapy* (eds. Baldwin and Byers, Academic Press, 1985), pp. 159-179,

224-266, all of which are incorporated herein in their entirety by reference for all purposes.)

Th delivery component of the immunotoxin will include the immunoglobulins discussed *supra*. Intact immunoglobulins or their binding fragments, such as Fab or Fv, are preferably used. Typically, the antibodies in the immunotoxins will be of the human IgM or IgG isotype, but other mammalian constant regions may be utilized as desired.

The therapeutic agents and compositions of the invention are preferably administered before commencement of reperfusion. Indeed, in situations where the likelihood of ischemia is predictable (*e.g.*, elective heart surgery), the therapeutic agents can be advantageously administered before the blockage to the blood supply occurs. Additional dosages of therapeutic agent may be administered throughout the reperfusion period and for several hours or days thereafter.

Therapeutic agents and compositions are administered in amount such as to prevent or at least detectably reduce the tissue damage and its complications that would otherwise result from reperfusion therapy. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend upon the severity of the disease and the general state of the patient's own immune system, but generally range from about 0.01 - 10 mg/kg body weight, preferably of 0.14 - 5 mg/kg. and most preferably of 0.3 - 3 mg/kg. The antibodies can be given as an intravenous bolus injection, *e.g.*, over 1 - 5 min., repeated injections of smaller doses, or as an intravenous infusion. The bolus injection is especially useful before commencing reperfusion therapy, or in an emergency. Further doses of antibodies can be repeated (*e.g.*, every 4 - 6 h) during and after reperfusion therapy at the same doses as described above to achieve optimal plasma levels of the antibody. Because many of the therapeutic agents of the invention comprise substantially or entirely human polypeptides, they can be tol rat d in high d sages.

The therapeutic agents and pharmaceutical compositions thereof of this invention are usually

administered parenterally, *i.e.*, subcutaneously, intramuscularly or, intravenously. Intravenous administrati n is preferr d. The agents can also be administered, typically for local application, by gavage or lavage, intraperitoneal injection, ophthalmic ointment, topical ointment, intracranial injection (typically into a brain ventricle), intrapericardiac injection, or intrabursal injection.

EXAMPLES

Example 1: Cross-Reactivity of hu DREG-200 with Feline PMNs

Binding of humanized MAb DREG-200 of IgG4 isotype (hu DREG-200) to L-selectin on cat neutrophils was determined by flow cytometric analysis employing a Becton-Dickinson FACS™ flow cytometer. Five ml of whole cat blood was collected into vacutainer tubes (containing 50 µl of 15% ethylene diamine tetracetic acid EDTA, Becton-Dickinson, Rutherford, NJ) and stored at 4°C. 200 µl of the anticoagulated blood was incubated with 40 µg/ml of hu DREG-200 or the control MAB isotype-matched, humanized ABL-364 (hu ABL-364) at 4°C for 30 min. Blood was centrifuged at 500g for 15 min at 4°C (Brinkmann Instruments Co.), the supernatant was discarded, and the pellet was washed with Dulbecco's PBS (Sigma Chemical Corp.) containing 0.2% bovine serum albumin (BSA) (Sigma Chemical Corp). The supernatant was removed following centrifugation and F(ab')2 fragments of a goat anti-mouse IgG-phycoerythrin conjugate (Tago, Inc., Burlingame, CA) (i.e., 1:100 dilution) was added as a secondary antibody for 30 min at 4°C. Following an additional wash with 0.2% BSA in PBS, the red cells were lysed utilizing a whole blood lysis kit (Coulter Immunology, Hialeah, FL). The remaining neutrophils were washed and fixed with 1% paraformaldehyde (Sigma Chemical Corp.). The binding of hu DREG-200 to PMNs was determined by appropriate gating of the neutrophil population based on cell size and granularity of the dot plot. Histograms were generated by cell number versus fluorescence intensity of at least 10,000 cells per sample.

It was found that 92.5% of the neutrophils stained positively with hu DREG-200 compared to 3±2% positive staining

with the control hu ABL-364. Binding of hu DREG-200 to feline neutrophils resulted in an increase of mean channel fluorescence of 82±3 compar d to 5±2 for hu ABL-364 ($p<0.01$). It is concluded that hu DREG-200 binds to L-selectin on cat neutrophils.

In an analogous experiment using the murine antibody, mouse DREG-200 (from which hu DREG-200 was derived), it was found that unstimulated cat neutrophils stained 75.0 +/- 9.3% positive compared with 5.1 +\- 0.3% positive staining for a control antibody. *See* Ma et al, *Circulation* 88:649-57 (1993) (which is incorporated by reference in its entirety for all purposes).

Example 2: Inhibition of Adherence by hu DREG-200 *In Vitro*

Peripheral blood was collected from the femoral artery of four cats and anticoagulated with citrate-phosphate-dextrose solution. PMNs were isolated and labeled as described above. Cats were subjected to 90 min of ischemia and 20 min of reperfusion. Hearts were removed and placed in warm, oxygenated K-H buffer. Both the ischemic-reperfused LAD and non-ischemic LCX coronary arteries were removed and cut in 2 to 3 mm segments. The arteries were then opened, and placed into a cell culture dish filled with 3 ml of K-H buffer. The labeled neutrophils (i.e., 1.2×10^6/ml) were incubated with different concentrations of hu DREG-200 (i.e., 5, 10, 20 or 40 μg/ml) or the control hu ABL-364 (40 μg/ml) for 10 min at 4°C. Following the incubation, the PMNs were washed twice with cold K-H solution. Neutrophils were added into K-H solution of the cell culture dishes containing the coronary arteries to yield a PMN count of 4×10^5/ml. After the incubation period, the coronary rings were removed and placed onto glass microscope slides. PMNs were counted using epifluorescence microscopy (Nikon Corp., Japan). Adherent neutrophils were counted on five fields from each vessel segment, averaged, and expressed as PMNs/mm^2.

Fig. 2 shows that increased adhesion of unstimulated PMN adherence to coronary artery endothelium following ischemia/reperfusion was significantly ($p<0.01$) inhibited by

10 to 40 μg/ml of hu DREG-200 in a concentration d pendent manner. By contrast, hu ABL-364 had no significant effect on PMN adherence to the ischemic-r perfused ndothelium. These results confirm that L-selectin, contributes significantly to the interaction between PMNs and the coronary endothelium post-reperfusion, and that this interaction can be blocked by hu DREG-200.

In an analogous experiment with the mouse DREG-200 antibody, similar results were obtained. Mouse DREG-200 inhibited PMN adherence to the endothelium in a concentration-dependent manner with a maximal inhibition of about 70% occurring at 20 μg/ml of mouse DREG-200. *See* Ma et al. (1993), *supra*.

Example 3: Effect of hu DREG-200 on Myocardial Injury Following Reperfusion

The effect of hu DREG-200 on the degree of actual myocardial salvage of ischemic tissue following reperfusion was investigated. Adult male cats (2.8-4.2 kg) were anesthetized with sodium pentobarbital (30 mg/kg, i.v.). An intratracheal cannula was inserted through a midline incision, and the cats were placed on intermittent positive-pressure ventilation (Harvard small animal respirator, Dover, MA). A polyethylene catheter was inserted into the right external jugular vein for additional pentobarbital infusion in order to maintain a surgical plane of anesthesia and for administration of antibodies. Another polyethylene catheter was inserted through the left femoral artery and positioned in the abdominal aorta for the measurement of mean arterial blood pressure (MABP) via a pressure transducer (Cobe Instruments, Lakewood, CO). After a midsternal thoracotomy, the anterior pericardium was incised, and a 3-0 silk suture was placed around the left anterior descending (LAD) coronary artery 8 to 10 mm from its origin. A high-fidelity catheter tip pressure transducer (Model MPC 500, with transducer control unit - Model TCB 500, Millar Instruments Inc., Houston, TX) was introduced into the 1 ft ventricle through the apical dimple. The catheter was positioned via observation of the LV pressure

and dP/dt wave forms and then secured in place by a silk suture. Standard lead II of the scalar electrocardiogram (ECG) was used to determine heart rate (HR) and ST-segm nt elevation. ST-segment elevations were determined by analysis of the ECG recording at 50 mm/sec every 20 min. The ECG, MABP, LVP and dP/dt were continuously monitored on a Hewlett-Packard 78304 A unit (Hewlett-Packard, Palo Alto, CA) and recorded on a Gould oscillographic recorder (Gould Inc., Cleveland, OH) every 20 min. The pressure-rate index (PRI), an approximation of myocardial oxygen demand, was calculated as the product of MABP and HR divided by 1000.

After completing all surgical procedures, the cats were allowed to stabilize for 30 minutes, at which time baseline readings of ECG, MABP, LVP and dP/dt were recorded. Myocardial ischemia (MI) was induced by tightening the initially placed reversible ligature around the LAD so that the vessel was completely occluded. This was designated as time point zero. Two mg/kg of hu DREG-200 (IgG4 isotype, as in previous examples) or a control hu ABL-364 (i.e., isotypematched humanized control IgG4 MAb) was given intravenously as a bolus 80 min after coronary occlusion (i.e., 10 min prior to reperfusion, R). Ten minutes later (i.e., after a total of 90 min ischemia, I) the LAD ligature was untied and the ischemic myocardium was reperfused for 4.5 h.

The cats were randomly divided into three major groups. Six sham MI + R cats received hu DREG-200 (2 mg/kg), six MI + R cats received the control hu ABL-364 (2 mg/kg), and six MI + R cats received hu DREG-200 (2 mg/kg). Sham MI + R cats were subjected to the same surgical procedures as MI + R cats except that the LAD coronary artery was not occluded.

At the end of the 4.5 h reperfusion period, the ligature around the LAD was again tightened. 20 ml of 0.5% Evans blue was rapidly injected into the left ventricle to stain the area of myocardium which was perfused by the pat nt coronary arteries. The area-at-risk was determined by negative staining. Immediately following this injection, the heart was rapidly excised and plac d in warmed, oxygenated K-H

solution. The left circumflex (LCX) and the LAD coronary arteries were isolated and removed for subsequent study of coronary ring vasoactivity and PMN adherence. The right ventricle, great vessels, and fat tissue were carefully removed, and the left ventricle was sliced parallel to the atrioventricular groove in 3 mm thick sections. The unstained portion of the myocardium (i.e., the total area-at-risk or ischemic) was separated from the Evans-blue-stained portion of the myocardium (i.e., the area-not-at-risk or non-ischemic). The area-at-risk was sectioned into small cubes and incubated in 0.1% nitroblue tetrazolium in phosphate solution at pH 7.4 and 37°C for 15 min. The tetrazolium dye forms a blue formazan complex in the presence of myocardial cells containing active dehydrogenases and their cofactors. The irreversibly injured or necrotic portion of the myocardium-at-risk which did not stain was separated from the stained portion of the myocardium (i.e., the ischemic but non-necrotic area). The three portions of the myocardium (i.e., non-ischemic, ischemic non-necrotic, and ischemic necrotic tissue) were subsequently weighed. Results were expressed as necrotic cardiac tissue area as a percentage of either the area-at-risk or of total left ventricular mass.

According to both of these criteria, cardiac tissue damage was significantly attenuated ($p<0.001$) in cats treated with hu DREG-200. Similar results have been obtained in an analogous experiment using the mouse DREG-200 antibody. *See* Ma et al. (1993), *supra*. For hu DREG-200, whereas about 30% of the jeopardized myocardium developed into necrotic tissue in the group treated with the control antibody, the amount of necrotic tissue was less than 15% ($p<0.01$) in the hu DREG-200 treated group, a decrease of 50-60%. *See* Fig. 3. There was no significant difference in the wet weights of the areas-at-risk expressed as a percentage of total left ventricle between the two ischemic groups (Fig. 3), indicating that a comparable ar a of myocardial ischemia occurred in both groups. Therefore, hu DREG-200 significantly protects against reperfusion injury.

The remarkable preservation of ischemic tissue by hu DREG-200 is further illustrated from measurements of plasma creatine kinase activity, a biochemical marker of myocardial injury. Arterial blood samples (2 ml) were drawn immediately before ligation and hourly thereafter. The blood was collected in polyethylene tubes containing 200 IU of heparin sodium. Samples were centrifuged at 2000 x g and 4°C for 20 min and the plasma was decanted for biochemical analysis. Plasma protein concentration was assayed using the biuret method of Gornall et al. (*J. Biol. Chem.* 177:751-766 (1949)). Plasma creatine kinase (CK) activity was measured using the method of Rosalki (*J. Lab. Clin Med.* 69:696-705 (1967)) and expressed as IU/μg protein.

In sham MI/R cats receiving hu DREG-200, the plasma CK activity increased slightly throughout the 6 hour observation period reaching a final value of 3.8 ± 0.9 IU/μg protein. In the two ischemic groups, plasma CK activity increased slightly during the period of myocardial ischemia. In cats receiving hu ABL-364, CK activity in circulating blood increased markedly within the first 30 min follow reperfusion and further increased during the remaining four hours of reperfusion. By contrast, ischemic cats treated with hu DREG-200 developed significantly lower plasma CK activities compared with ischemic cats receiving the hu ABL-364 ($p<0.05$). *See* Fig. 4. The effect was sustained over the entire reperfusion period further evidencing the substantial protection conferred by hu DREG-200 against myocardial reperfusion injury.

Example 4: Effect of hu DREG-200 on Cardiac Function

Left ventricular pressure (LVP) and the first derivative of LVP, dP/dt max, an index of myocardial contractility, were measured by a catheter tip manometer inserted in the left ventricular cavity. All three groups sh wed comparabl initial values for these cardiac variables. In the sham MI group there wer no significant changes in dP/dt max over the entire six hour experimental period. However, in both MI/R groups, dP/dt max decreased upon

occlusion of the LAD to about 65%. In cats given hu ABL-364, contractility did not significantly recover. However, in hu DREG-200 treated MI-R cats, dP/dt max recovered to c ntrol values three hours following reperfusion. Hence, after 4.5 hours of reperfusion, dP/dt max was significantly lower in hu ABL-364 treated cats than in hu DREG-200 treated cats ($p<0.01$) (Fig. 6). These results indicate that hu DREG-200 not only prevented myocardial necrosis following reperfusion of the ischemic myocardium, but this myocardial salvage was also translated into an improvement in mechanical performance of the heart.

Example 5: Effect of hu DREG-200 on Coronary Endothelial Dysfunction

Since endothelial dysfunction is an early and critical event in reperfusion injury, endothelial dysfunction was tested by comparing vasoactivity of isolated coronary artery rings to the endothelium-dependent vasodilators, ACh and A-23187, and to the endothelium-independent vasodilator, $NaNO_2$.

Both LAD and LCX coronary segments were removed and placed into warmed K-H buffer. Isolated coronary vessels were cleaned and cut into rings 2 to 3 mm in length. The rings were then mounted on stainless steel hooks, suspended in 10 ml tissue baths, and connected to FT-03 force displacement transducers (Grass Instrument Co., Quincy, MA) to record changes in force on a Grass Model 7 oscillographic recorder. The baths were filled with 10 ml of K-H buffer and aerated at 37°C with a gas mixture of 95% O_2 and 5% CO_2. Coronary rings were initially stretched to give a preload of 0.5 grams of force and equilibrated for 60-90 minutes. During this period, the K-H buffer in the tissue baths was replaced every 15 min. After equilibration, the rings were then exposed to 100 nM U-46619 (9,11-epoxymethano-PGH_2, Biomol Research Laboratories, Plymouth Meeting, PA), a thromboxan A_2 mimetic, to generate about 0.5 g of developed force. Once a stable contraction was obtained, acetylcholine (ACh), an endothelium-dependent vasodilator, was added to the bath in cumulative

conc ntrations of 0.1, 1, 10, and 100 nM. After the response stabilized, the rings were washed and allowed to equilibrate to baseline once again. The procedur was repeated with another endothelium-dependent vasodilator, A-23187, (1, 10, 100 and 1000 nM) and then again with an endothelium-independent vasodilator, acidified $NaNO_2$ at pH 2, (0.1, 1, 10, and 100 μM). Saline (i.e., 0.9% NaCl) titrated to pH 2 did not relax coronary arteries at all. Relaxation was calculated as % relaxation from the U46619 induced contraction.

Fig. 7 summarizes the vasorelaxant responses to ACh, A-23187, and NaNO2 of ischemic reperfused LAD coronary rings or non-ischemic LCX obtained from sham MI + I R cats or MI + R cats receiving either hu ABL-364 or hu DREG-200. Coronary vascular rings isolated from sham MI + R cats exhibited full relaxation to the endothelium-dependent vasodilator ACh and A-23187 as well as to the endothelium-independent vasodilator, $NaNO_2$. Rings from ischemic reperfused cats receiving the control MAb also showed unchanged relation to $NaNO_2$, but relaxation to ACh and A-23187 was significantly retarded, indicating endothelial dysfunction. By contrast, relaxation to ACh and A-23187 in rings obtained from cats treated with hu DREG-200 was significantly preserved. Control LCX coronary rings isolated from all three groups relaxed fully to both the endothelium-dependent vasodilators (i.e., ACh and A23187) and the endothelium-independent vasodilator, $NaNO_2$. Thus, hu DREG-200 significantly protects against endothelial dysfunction observed in coronary artery rings subjected to myocardial ischemia and reperfusion.

In an analogous experiment, similar results have been obtained using the mouse DREG-200 antibody. *See* Ma et al. (1993), *supra*.

Example 6: Inhibition of Neutrophil Accumulation in the Ischemic-Reperfused Area by hu DREG-200

The protective activity of hu DREG-200 is further manifested by its capacity to prevent accumulation of neutrophils in the ischemic region during reperfusion. Neutrophil accumulation is thought to be one of the major

m chanisms responsible for reperfusion injury. Neutrophil accumulation was assayed from myeloperodixase (MPO) activity, an enzyme occurring virtually exclusively in neutrophils, in the non-ischemic, ischemic and necrotic portions of the myocardium.

MPO activity was determined using the method of Bradley et al., *J. Invest. Dermatol.* 78:206-209 (1992) as modified by Mullane et al., *J. Pharmacol. Methods* 14:157-167 (1985). The myocardium was homogenized in 0.5% HTAB (Sigma Chemical Co., St. Louis, MO) and dissolved in 50 mM potassium phosphate buffer at pH 6.0 using a Polytron (PCU-2) homogenizer. Homogenates were centrifuged at 12,500 x g, 2°C for 30 min. The supernatants were than collected and reacted with 0.167 mg/ml of O-dianisidine dihydrochloride (Sigma Chemical Co., St. Louis, MO) and 0.0005% H_2O_2 in 50 mM phosphate buffer at pH 6.0. The change in absorbance was measured spectrophotometrically at 460 nm. One unit of MPO is defined as that quantity of enzyme hydrolyzing 1 mmol of peroxide/min at 25°C.

Fig. 5 shows that in the non-ischemic myocardium (i.e., area-not-at-risk), MPO activity was equally low in both MI groups. However, MI cats receiving only the control antibody, hu ABL-364, exhibited a marked increase in MPO activity in the ischemic region. MPO activity increased in the necrotic portion to 0.8 ± 0.2 IU/μg protein. In contrast, hu DREG-200-treated ischemic cats exhibited a significantly lower MPO activity in both ischemic non-necrotic myocardial tissue (0.12 ± 0.10 IU/μg protein, $p<0.05$) and necrotic myocardial tissue (0.20 ± 0.12 IU/μg protein, $p<0.01$). These results indicate that hu DREG-200 retards neutrophil accumulation in the ischemic-reperfused myocardium.

In an analogous experiment, similar results have been obtained using the mouse DREG-200 antibody. *See* Ma et al. (1993), *supra*.

Example 7: Absence of Side Effects from hu DREG-200 Treatment

In cats subjected to sham myocardial ischemia and reperfusion, intravenous administration of hu DREG-200 (2 mg/kg) had no detectable effect on any of the measured hemodynamic or electrocardiographic variables. In the two groups of MI + R cats (receiving hu DREG-200 or control antibody), there were no significant differences in any of the variables observed before coronary occlusion. A few minutes after LAD occlusion, the ST-segment of the ECG became significantly elevated in both groups. After reperfusion, the ST-segment decreased to nearly control values, indicating that effective reperfusion had occurred. There was no significant difference in peak ST-segment elevation between the two MI + R groups (0.25 ±0.04 mV vs. 0.20 ± 0.05 mV for the hu DREG-200 and hu ABL-364 groups, respectively), indicating that the ischemic insult was similar in these two MI + R groups. At reperfusion, there was a noticeable increase in the incidence of premature ventricular contractions in all cats. Three cats in the MI + R group treated with hu ABL-364 and two in the hu DREG-200 group developed ventricular fibrillation prior to antibody treatment which was successfully converted to a normal sinus rhythm by using a DC electronic defibrillator (Sanborn Co, Waltham, MA). However, there was no obvious overall difference between the MI + R groups in the number of PVCs occurring post-reperfusion, indicating that hu DREG-200 did not appear to exert any overt anti-arrhythmic effect. In both groups of MI + R cats, the PRI decreased significantly after coronary occlusion and gradually returned to nearly control values after reperfusion. There was no significant differences between the two MI + R groups at any of the hourly PRI readings, suggesting hu DREG-200 did not exert a systemic hemodynamic effect (i.e., reduced myocardial oxygen demand) that could account for the cardioprotection.

As a further control to determine whether hu DREG-200 exerted any leukopenic effects, circulating white blood cells (WBC) were counted over the experimental period. Peripheral WBCs were counted 5 min before coronary occlusion, 5 min before reperfusion, and 30, 150 and 270 min after

reperfusion. WBC counts did not change significantly over the c urse of the experiment in either the sham MI group, the hu ABL-364 group or the hu DREG-200 treated group. Furthermore, there were no significant differences in total leukocyte counts among the three groups of cats at any time. All samples checked exhibited 60-65% PMNs of total WBCs. These results show that hu DREG-200 does not produce leukopenia.

The above examples are provided to illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill and are encompassed by the claims of the invention. All publications, patents and patent applications cited in the application are hereby incorporated by reference in their entirety for all purposes to the same extent as if each were individually denoted as being so incorporated by reference.

WHAT IS CLAIMED IS:

1. A method for prevention or treatment of reperfusion injury in a patient, the method comprising administering a therapeutically effective dose of an agent that specifically binds to L-selectin thereby inhibiting adherence of leukocytes to endothelial cells.

2. The method of claim 1, wherein the patient is human.

3. The method of claim 2, wherein the agent is an antibody.

4. The method of claim 3, wherein the antibody is a monoclonal antibody.

5. The method of claim 4, wherein the monoclonal antibody is a humanized antibody.

6. The method of claim 4, wherein the monoclonal antibody is a human antibody.

7. The method of claim 5, wherein the monoclonal antibody competes with an antibody designated hu DREG-200 for specific binding to L-selectin.

8. The method of claim 6, wherein the monoclonal antibody competes with an antibody designated hu DREG-200 for specific binding to L-selectin.

9. The method of claim 7, wherein the humanized antibody is of IgG4 isotype.

10. The method of claim 3, wherein the antibody is a Fab or $(Fab')_2$ fragment.

11. The method of claim 5, further comprising the step of administering a therapeutically effective dose of a thrombolytic agent.

12. The method of claim 6, further comprising the step of administering a therapeutically effective dose of a thrombolytic agent.

13. The method of claim 5, further comprising the step of administering a therapeutically effective dose of an antibody specific for CD18.

14. The method of claim 6, further comprising the step of administering a therapeutically effective dose of an antibody specific for CD18.

15. The method of claim 3, wherein the patient is suffering from myocardial ischemia.

16. The method of claim 3, wherein the patient is undergoing angioplasty.

17. The method of claim 3, wherein the patient is suffering from renal, cerebral, splenic or hepatic ischemia.

18. A method for prevention or treatment of reperfusion injury in a patient, the method comprising administering a therapeutically active dose of a monoclonal antibody that specifically binds to an epitope of L-selectin responsible for adherence of leukocytes to endothelial cells.

19. The method of claim 18, wherein the monoclonal antibody competes with an antibody designated hu DREG-200 for specific binding to L-selectin.

20. The method of claim 19, wherein the patient is suffering from myocardial ischemia.

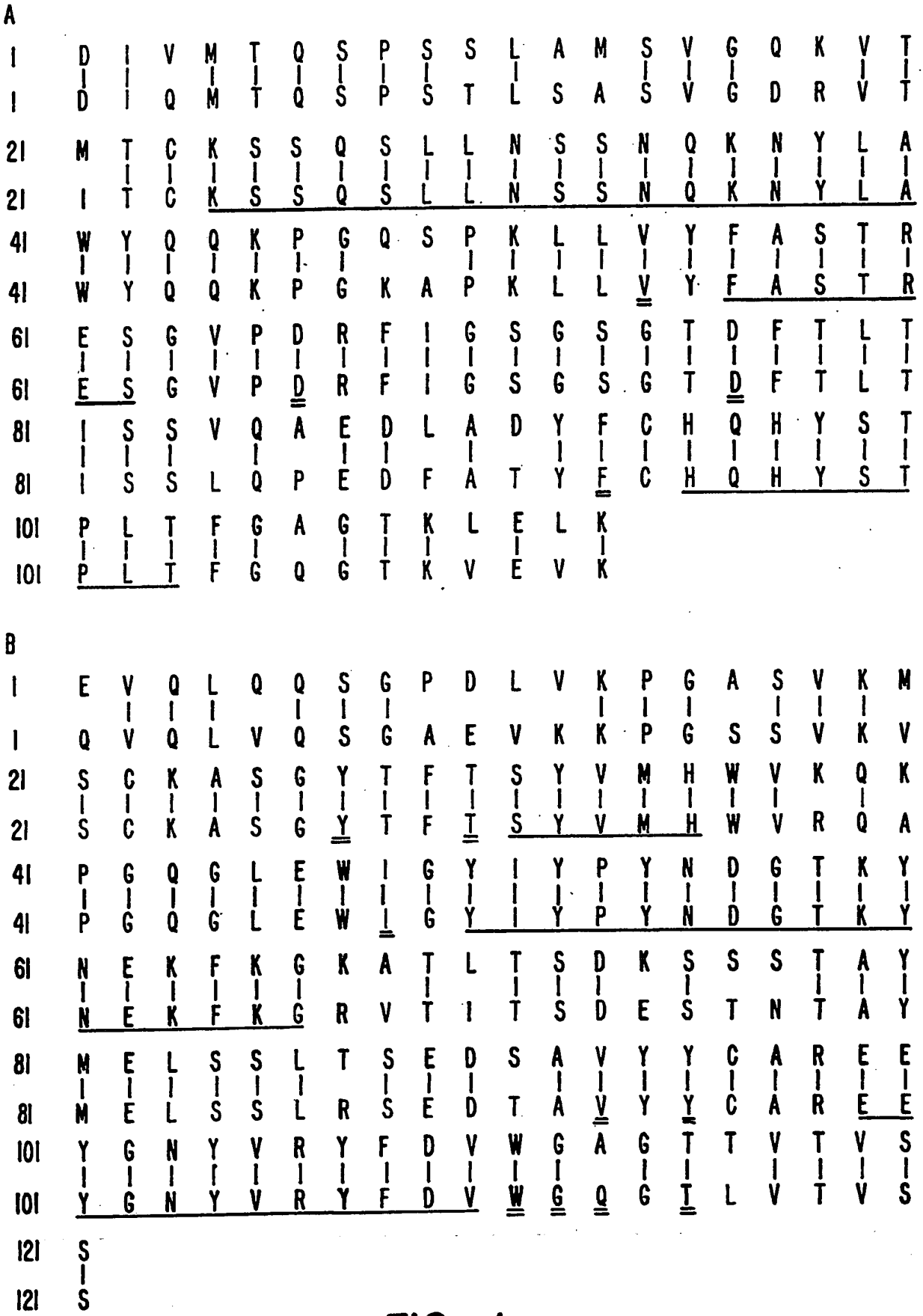

FIG. 1.

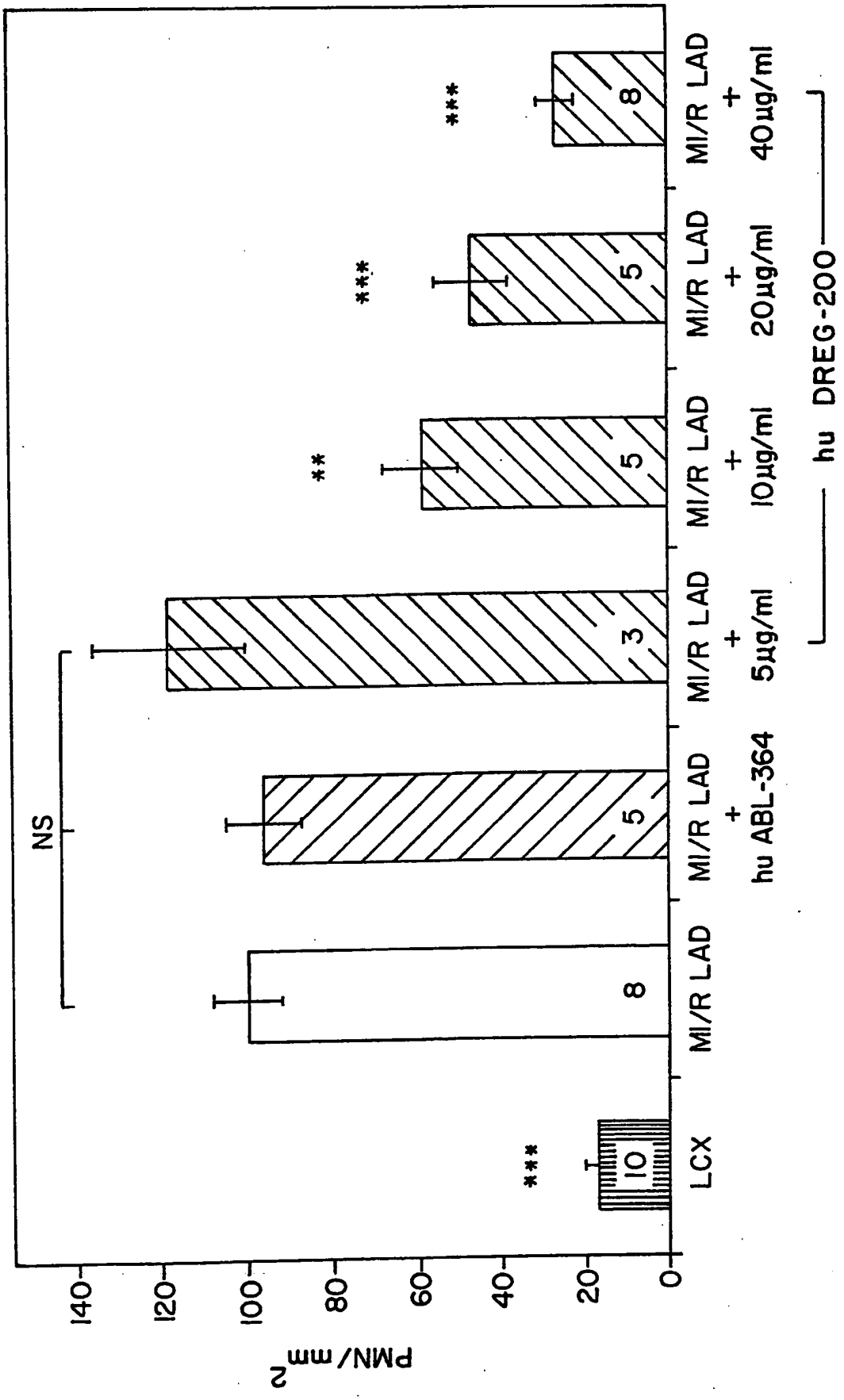
PMN/ mm2
0
20
40
60
80
100
120
140
LCX
MI/R LAD
MI/R LAD + hu ABL-364
MI/R LAD + 5µg/ml
MI/R LAD + 10µg/ml
MI/R LAD + 20µg/ml
MI/R LAD + 40µg/ml
hu DREG-200
10
8
5
3
5
5
8
NS
***
**
***
***

FIG. 2.

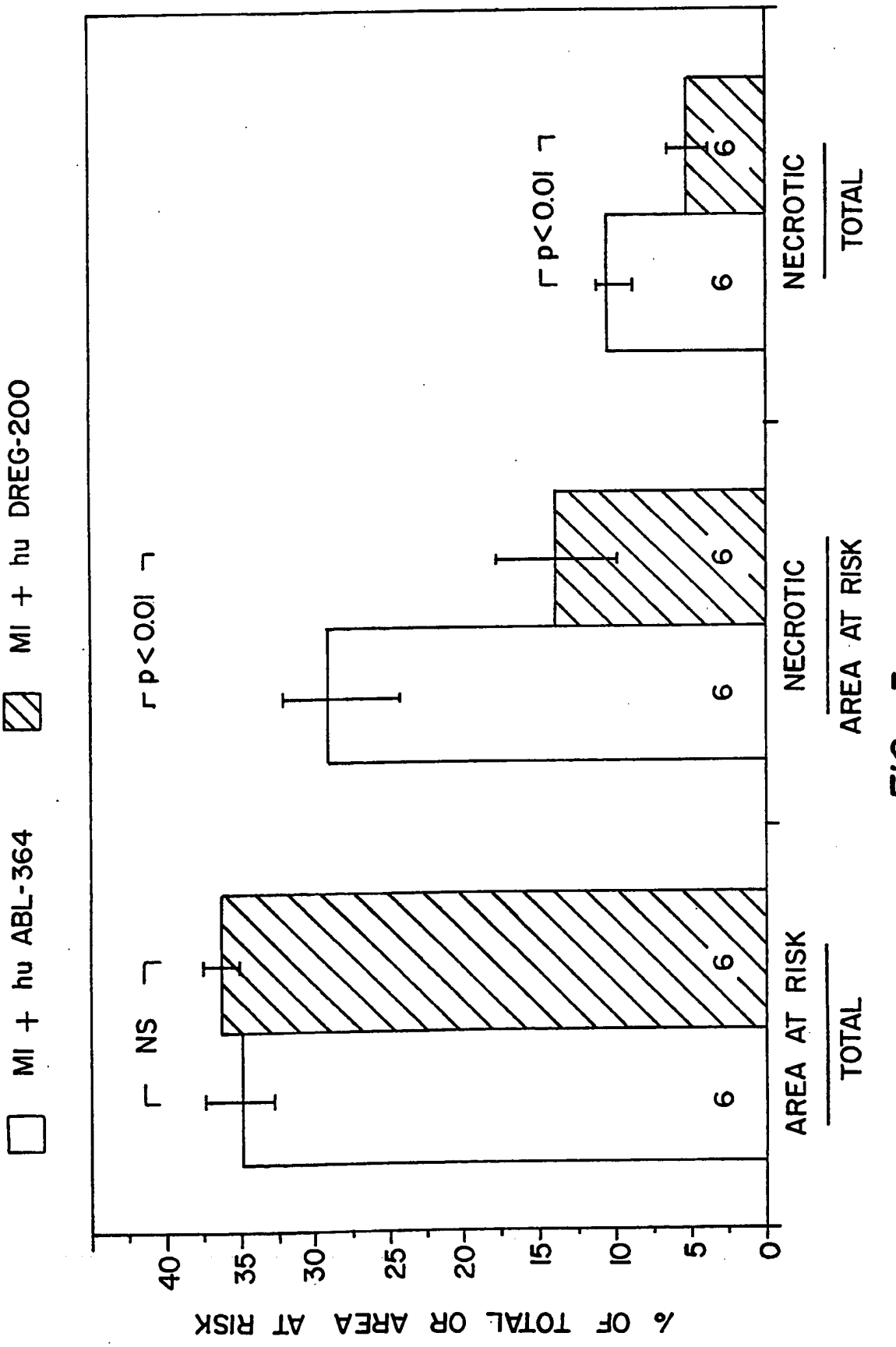
% OF TOTAL OR AREA AT RISK
0
5
10
15
20
25
30
35
40
MI + hu ABL-364
MI + hu DREG-200
NS
p<0.01
p<0.01
6
6
6
6
AREA AT RISK
TOTAL
NECROTIC
AREA AT RISK
NECROTIC
TOTAL

FIG. 3.

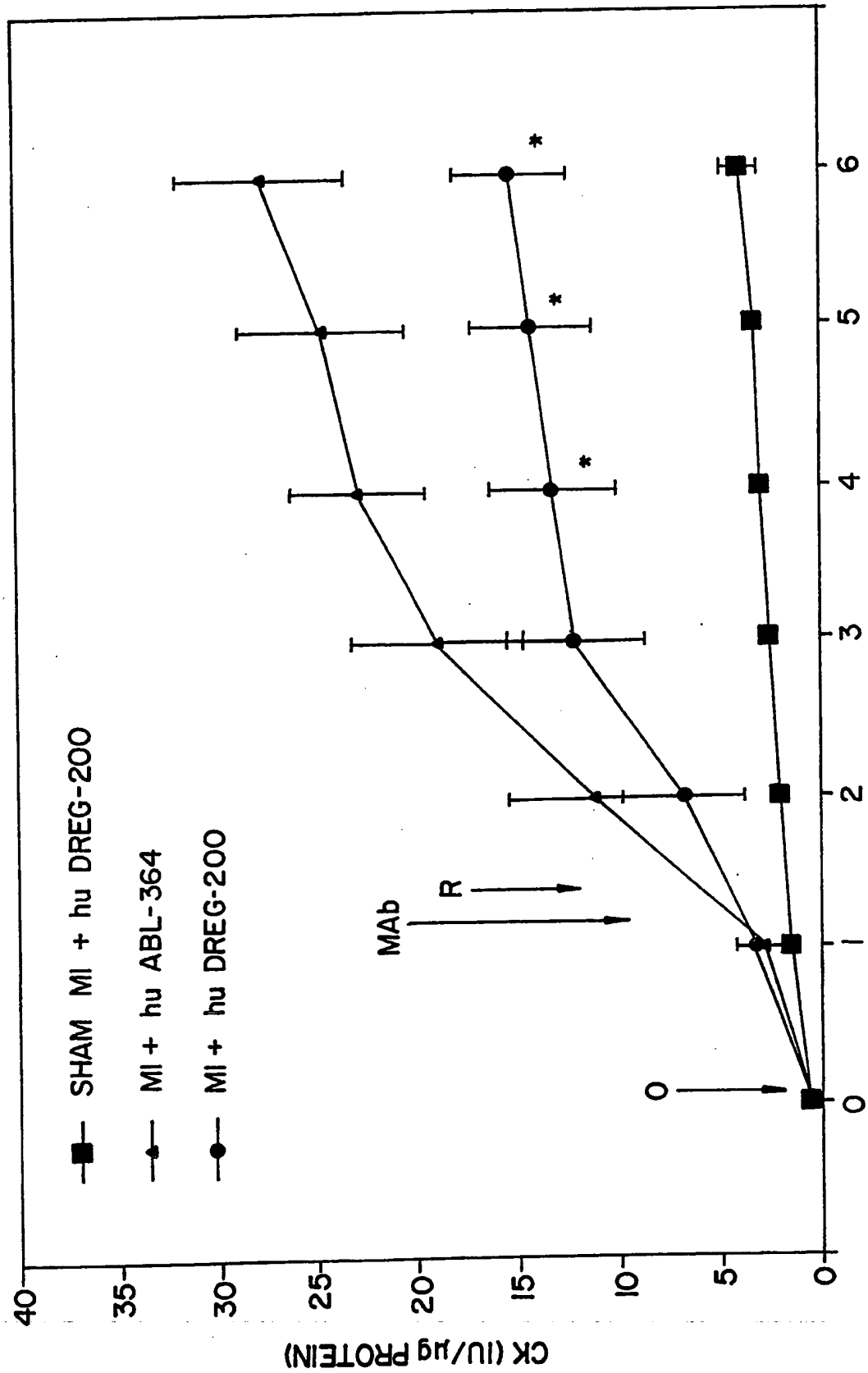
CK (IU/µg PROTEIN)
40
35
30
25
20
15
10
5
0
TIME (HOURS)
0
1
2
3
4
5
6
MAb
R
O
SHAM MI + hu DREG-200
MI + hu ABL-364
MI + hu DREG-200
*
*
*

FIG. 4.

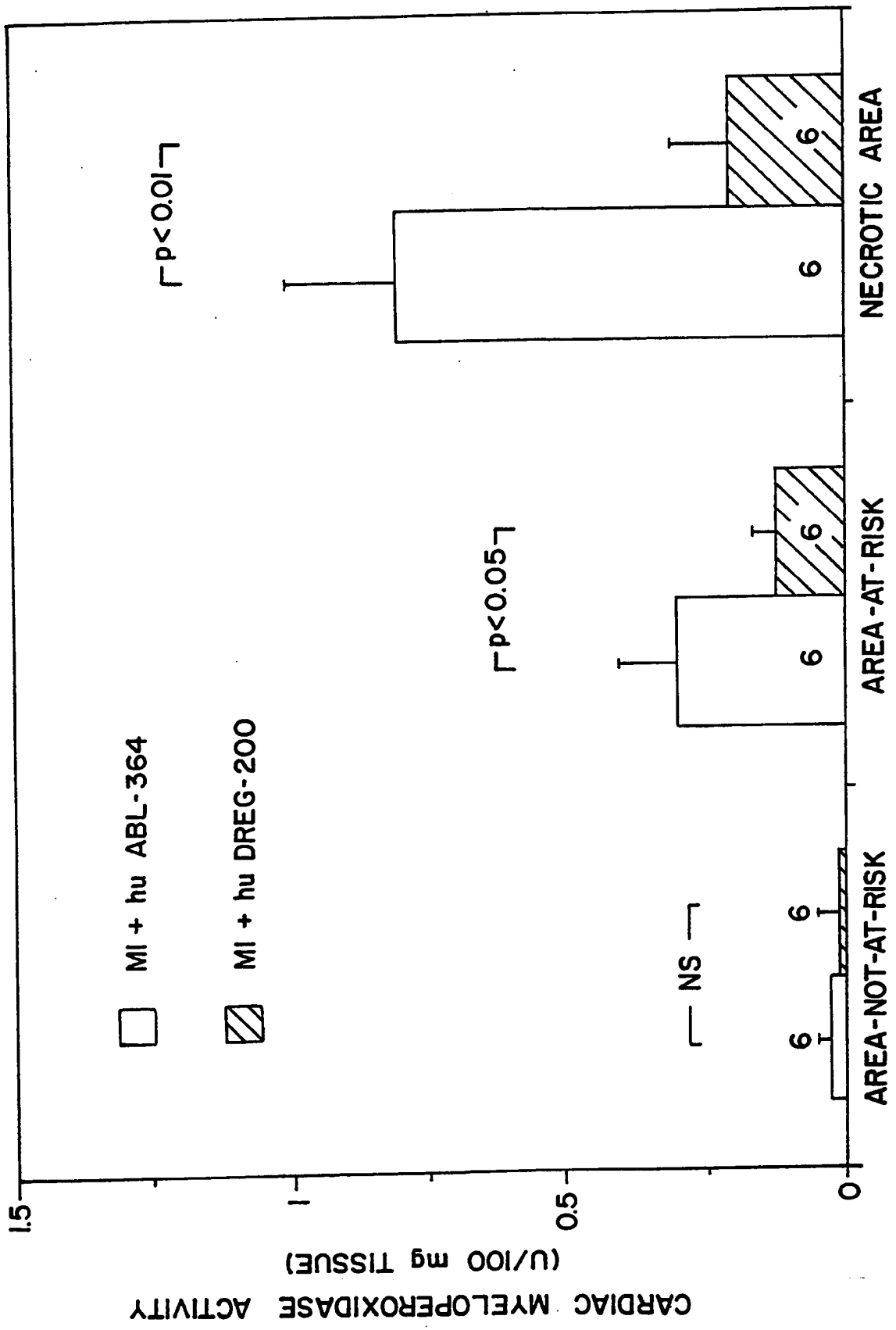
CARDIAC MYELOPEROXIDASE ACTIVITY
(U/100 mg TISSUE)
1.5
1
0.5
0
MI + hu ABL-364
MI + hu DREG-200
NS
p<0.01
p<0.05
6
6
6
6
AREA-NOT-AT-RISK
AREA-AT-RISK
NECROTIC AREA

FIG. 5.

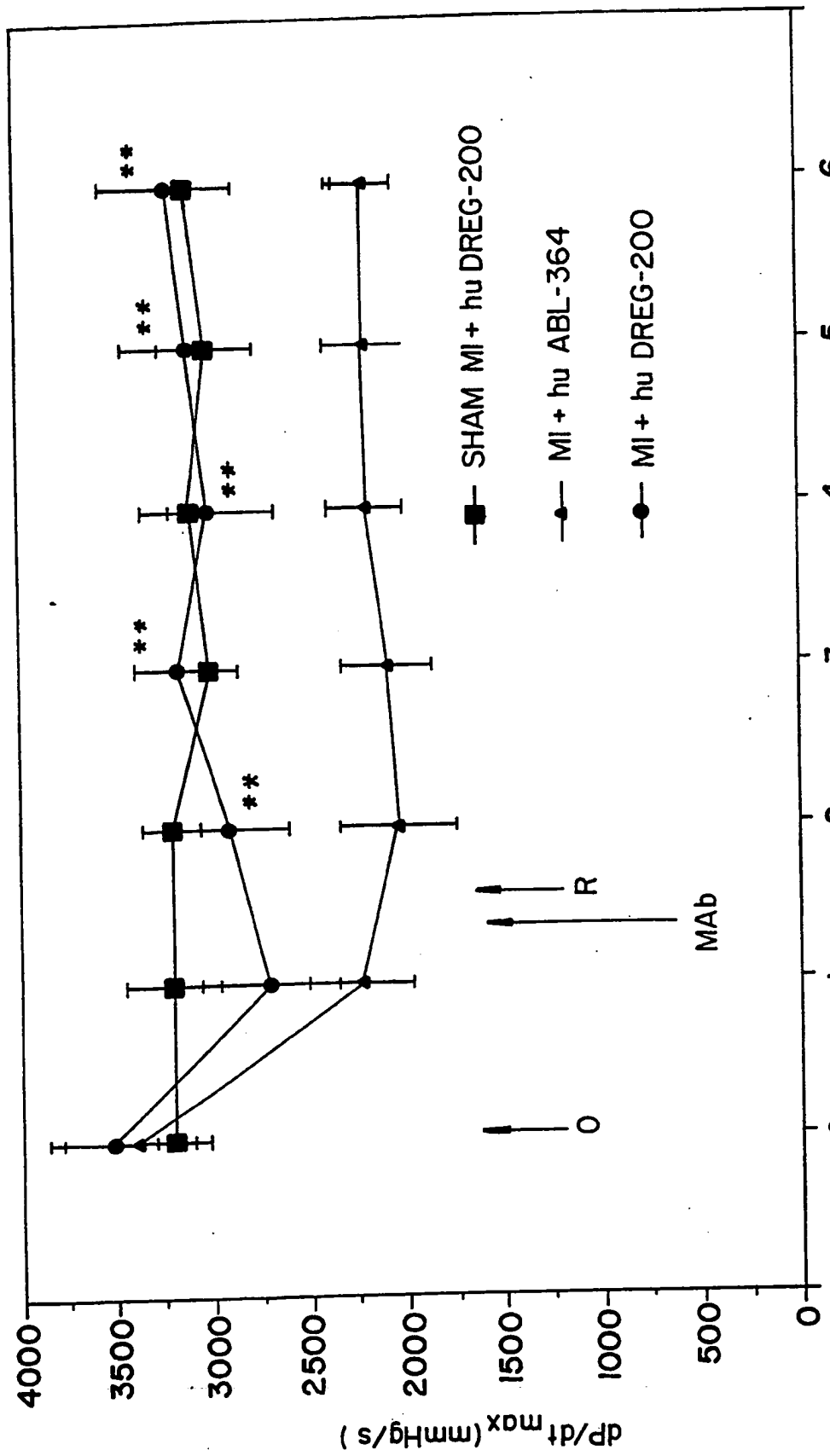
dP/dt max (mmHg/s)
4000
3500
3000
2500
2000
1500
1000
500
0
0
1
2
3
4
5
6
TIME (HOURS)
SHAM MI + hu DREG-200
MI + hu ABL-364
MI + hu DREG-200
R
MAb
O
**

FIG. 6.

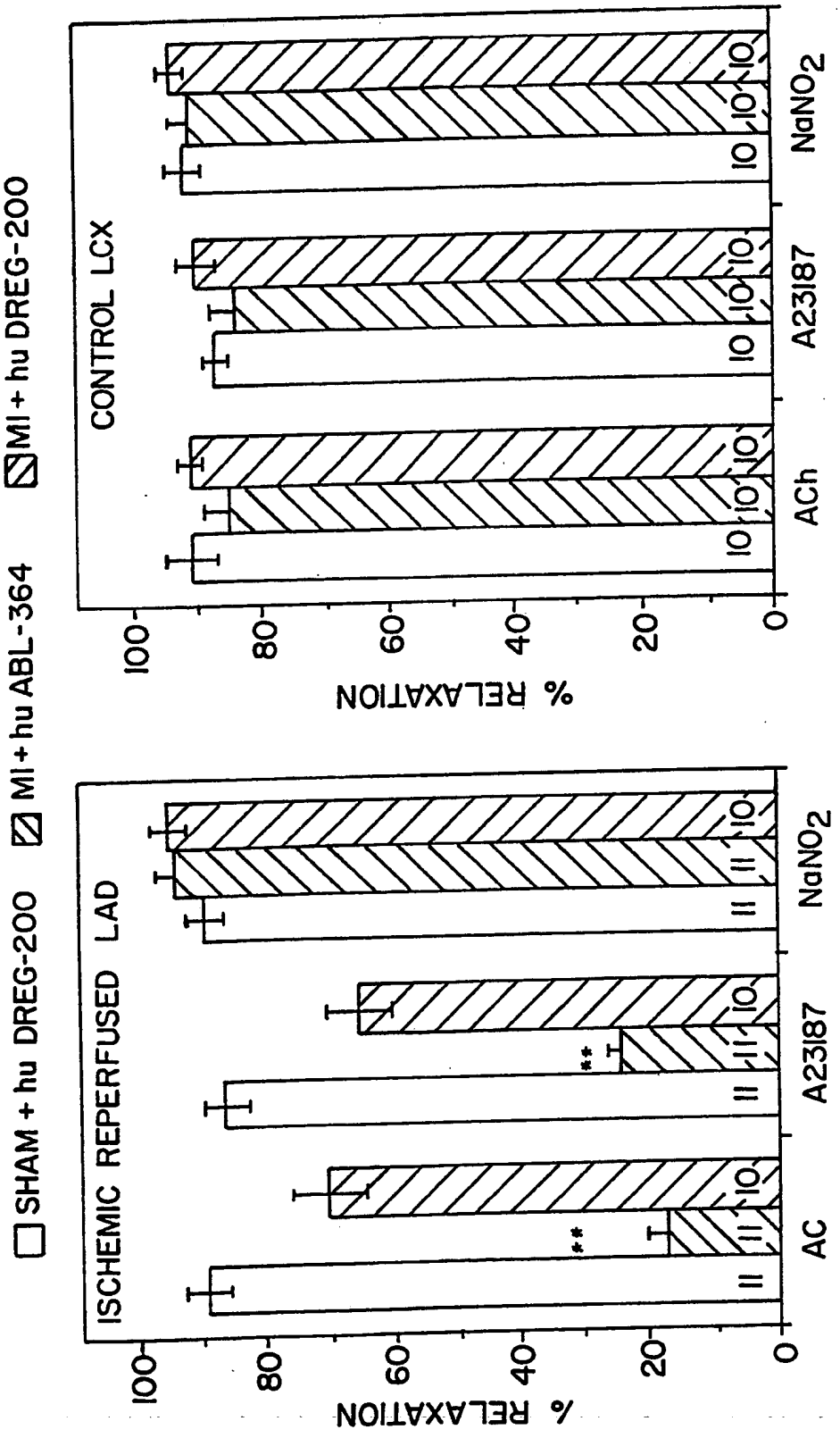
SHAM + hu DREG-200
MI + hu ABL-364
MI + hu DREG-200
ISCHEMIC REPERFUSED LAD
CONTROL LCX
% RELAXATION
0
20
40
60
80
100
AC
A23187
NaNO2
ACh
A23187
NaNO2
**
**

FIG. 7.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/13828

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 39/395; C07K 16/00, 16/18, 16/28
US CL :Pleas See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/130.1, 133.1, 139.1, 141.1, 144.1, 152.1, 153.1, 154.1; 530/387.3, 388.7, 388.73, 388.75

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, BIOSIS, EMBASE, MEDLINE, CHEM ABSTRACTS , WPI
search terms: L-selectin, LAM, DREG-200, CD18

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,797,277 (AFORS) 10 JANUARY 1989, SEE ENTIRE DOCUMENT.	1-20
Y	WO, A, 90/07861 (QUEEN ET AL.) 26 JULY 1990, SEE ENTIRE DOCUMENT.	1-20
Y	SCIENCE, VOLUME 252, ISSUED 21 JUNE 1991, WALDMANN, "MONOCLONAL ANTIBODIES IN DIAGNOSIS AND THERAPY", PAGES 1657-1662, SEE ENTIRE DOCUMENT.	1-20
Y	IMMUNOLOGICAL REVIEWS, VOLUME 114, ISSUED 1990, CARLOS ET AL., "MEMBRANE PROTEINS INVOLVED IN PHAGOCYTE ADHERENCE TO ENDOTHELIUM", PAGES 5-21, SEE ENTIRE DOCUMENT.	1-20

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
20 FEBRUARY 1995

Date of mailing of the international search report
13 MAR 1995

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Authorized officer
PHILLIP GAMBEL
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BLOOD, VOLUME 78, NUMBER 3, ISSUED 01 AUGUST, 1991, KISHIMOTO ET AL., "ANTIBODIES AGAINST HUMAN NEUTROPHIL LECAM-1 (LAM-1/LEU-8/DREG-56 ANTIGEN) AND ENDOTHELIAL CELL ELAM-1 INHIBIT A COMMON CD18-INDEPENDENT ADHESION PATHWAY IN VITRO, PAGE 805-811, SEE ENTIRE DOCUMENT.	1-20
Y	PROC. NATL. ACAD. SCI. USA, VOLUME 88, ISSUED 1991, VON ANDRIAN ET AL., "TWO-STEP MODEL OF LEUKOCYTE-ENDOTHELIAL CELL INTERACTION IN INFLAMMATION: DISTINCT ROLES FOR LECAM-1 AND THE LEUKOCYTE β_2 INTEGRINS *IN VIVO*", PAGES 7538-7542, SEE ENTIRE DOCUMENT.	1-20
Y	PROC. NATL. ACAD. SCI. USA, VOLUME 87, ISSUED MARCH 1990, KISHIMOTO ET AL., "IDENTIFICATION OF A HUMAN PERIPHERAL LYMPH NODE HOMING RECEPTOR: A RAPIDLY DOWN-REGULATED ADHESION MOLECULE", PAGES 2244-2248, SEE ENTIRE DOCUMENT.	1-20

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/130.1, 133.1, 139.1, 141.1, 144.1, 152.1, 153.1, 154.1; 530/387.3, 388.7, 388.73, 388.75